Biomedical and Pharmaceutical Sciences Faculty Publications

Biomedical and Pharmaceutical Sciences

2014

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Differential *Fmo3* Gene Expression in Various Liver Injury Models Involving Hepatic Oxidative Stress in Mice

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Abstract

Flavin-containing monooxygenase-3 (FMO3) catalyzes metabolic reactions similar to cytochrome P450 monooxygenase however, most metabolites of FMO3 are considered non-toxic. Recent findings in our laboratory demonstrated *Fmo3* gene induction following toxic acetaminophen (APAP) treatment in mice. The goal of this study was to evaluate *Fmo3* gene expression in diverse other mouse models of hepatic oxidative stress and injury. *Fmo3* gene regulation by Nrf2 was also investigated using Nrf2 knockout (Nrf2 KO) mice. In our studies, male C57BL/6J mice were treated with toxic doses of hepatotoxicants or underwent bile duct ligation (BDL, 10d). Hepatotoxicants included APAP (400 mg/kg, 24 to 72h), alpha-naphthylisothiocyanate (ANIT; 50 mg/kg, 2 to 48h), carbontetrachloride (CCl_4; 10 or 30 μL/kg, 24 and 48h) and allyl alcohol (AlOH; 30 or 60 mg/kg, 6 and 24h). Because oxidative stress activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2), additional studies investigated *Fmo3* gene regulation by Nrf2 using Nrf2 knockout (Nrf2 KO) mice. At appropriate time-points, blood and liver samples were collected for assessment of plasma alanine aminotransferase (ALT) activity, plasma and hepatic bile acid levels, as well as liver Fmo3 mRNA and protein expression. Fmo3 mRNA expression increased

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significantly by 43-fold at 12h after ANIT treatment,and this increase translates to a 4-fold change in protein levels. BDL also increased Fmo3 mRNA expression by 1899-fold, but with no change in protein levels. Treatment of mice with CCl_4decreased liver *Fmo3*gene expression, whileno change in expression was detected with AlOH treatment. Nrf2 KO mice are more susceptible to APAP (400 mg/kg, 72h) treatment compared to their wild-type (WT) counterparts, which is evidenced by greater plasma ALT activity. Fmo3 mRNA and protein expression increased in Nrf2 KO mice after APAP treatment. Collectively, not all hepatotoxicantsthat produce oxidative stress alter *Fmo3*gene expression. Along with APAP, toxic ANIT treatment in mice markedly increased*Fmo3* gene expression. While BDL increased Fmo3 mRNA expression, protein level did not change. The discrepancy with *Fmo3* induction in cholestatic models, ANIT and BDL, is not entirely clear. Results from Nrf2 KO mice with APAP suggest that the transcriptional regulation of *Fmo3* during liver injury may not involve Nrf2.

Keywords

Flavin-containing monoxygenase-3; Hepatotoxicants; Acetaminophen; Alpha-naphthylisothiocyanate; Bile duct ligation; Carbon tetrachloride; Allyl alcohol; Oxidative stress; Nrf2

1. Introduction

Drug-induced liver injury (DILI) is a significant challenge for both drug development and clinical care. It accounts for more than 50% of all acute liver failure cases in the U.S. (Larson et al., 2005; W. M. Lee, 2010). Many chemicals, such as acetaminophen (APAP), carbontetrachloride (CCl_4) and allyl alcohol (AlOH) have been used to model hepatotoxicity relevant to human exposure. Alpha-naphthylisothiocyanate (ANIT) and bile duct ligation (BDL) on the other hand are used to model cholestasis, a pathological condition caused by impairment of hepatic bile flow. While ANIT produces intrahepatic cholestasis, BDL produces extrahepatic cholestasis. With APAP and CCl_4, the parent compound is metabolized by cytochrome P450 (CYP) to generate reactive metabolites, N-acetyl-p-benzoquinone imine (NAPQI) and tricholoromethyl radical ($^{\cdot}CCl_3$), respectively.AlOHin turn is metabolized in the liver by alcohol dehydrogenase to its reactive metabolite, acrolein. The glutathione adductof acrolein is converted by CYPs to glycidaldehyde. Toxicity resulting from these reactive metabolites is multifactorial and includes lipid peroxidation, generation of oxidative stress, altered cellular redox status and protein adduct formation(Burcham & Fontaine, 2001; Cohen et al., 1997; Jaeschke et al., 2012; Ohno et al., 1985; Tom et al., 1984). During cholestasis resulting from either BDL or ANIT treatment, increase in bile acid concentration stimulates production of reactive oxygen species eventually leading to hepatocellular necrosis and apoptosis (Sokol et al., 1995; Trauner et al., 1998).

The role of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) role as a master defense against hepatotoxicity produced by various chemicals has been investigated in several studies. Nrf2 belongs to the cap 'n' collar family of transcription factors that promotes transcription of a battery of cytoprotective genes(Aleksunes & Manautou, 2007; Kensler et al., 2007). Under

basal conditions, Nrf2 is largely bound to the cytoskeletal anchoring protein Kelch-like ECH-associated protein 1 (Keap1) also known as cytosolic Nrf2 inhibitor in the cytoplasm.In response to oxidative stress, Nrf2 is released from Keap1 andtranslocatesto the nucleus. In the nucleus, Nrf2 binds to the GTGACA***GC core sequence of the antioxidant response element (ARE) (Rushmore et al., 1991) and promotes ARE-mediated antioxidant gene expression.

A low toxic APAP dose causes nuclear accumulation of Nrf2 in mouse liver, which is accompanied by increased expression of Nrf2 dependent cytoprotectivegenes such as heme oxygenase-1 (*Hmox1*), NAD(P)H:quinone oxidoreductase-1 (*Nqo1*) and glutamate cysteine ligase catalytic subunit (*Gclc*) (Aleksunes et al., 2005; Aleksunes et al., 2006; Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004). Similar results have been reported with ANIT, BDL, CCl$_4$ and AlOH, other models of hepatic oxidative stress used in the present study(Aleksunes et al., 2005; Aleksunes et al., 2006; Liu et al., 2013; Randle et al., 2008; Tanaka et al., 2009). On the other hand, Nrf2 KO mice are more susceptible to APAP-induced liver injury compared to their wild-type counterparts (Chan et al., 2001; Enomoto et al., 2001). Likewise, Nrf2 KO mice arealso more susceptible to CCl$_4$- andAlOH-induced hepatoxicity compared towild-type mice (Liu et al., 2013). However, Nrf2 KO mice do not exhibit any difference in susceptibility to either BDL or ANIT treatment(Tanaka et al., 2009; Weerachayaphorn et al., 2012). This response is attributed to the adaptive compensatory changesinvolving nuclear transcription factors, including Fxr, Shp, Pxr andHnf1α, efflux bile acid transporters, altered GSH levels and bile flow rates in Nrf2 KO mice (Tanaka et al., 2009; Weerachayaphorn et al., 2012). Collectively, the models of hepatic injury selected for the current study not only result in hepatic oxidative stress but also activate the Nrf2-Keap1 regulatory pathway.

Despite *Fmo3* being considered non-inducible, studies with aryl hydrocarbon receptor (AhR) agonists in mice revealed liver *Fmo3* gene induction (Celius et al., 2008; Celius et al., 2010). A recent gene array analysis performed in our laboratory also demonstrated *Fmo3* gene induction in the APAP autoprotection mouse model (mice receiving a low hepatotoxic APAP dose that become resistant to a subsequent higher APAP dose)(O'Connor et al., 2014).Unlike with AhR agonists that result in marginal increases in Fmo3 protein expression in mouse liver, we showed significant increases in Fmo3 protein levels by 15-fold in APAP autoprotectedmice(Rudraiah et al., 2014). *Fmo3* induction by other hepatotoxicants that produce oxidative stress is not currently known.

In human liver, transcription factors regulating constitutive *FMO3*expression as well as those involved in developmental expression pattern have been extensively studied (Klick & Hines, 2007; Klick et al., 2008; Shimizu et al., 2008). Because the mammalian FMOs were considered non-inducible by xenobiotics (Cashman & Zhang, 2002; Krueger & Williams, 2005), the transcriptional regulation of FMO involving stress-activated transcription factorsor receptors that bind ligands andinteract with DNAwas not studied as other forms of regulation. Thus, little is known about the transcriptional regulation of *Fmo3* in response to toxicant exposure. Recently, Celius et al. (2010) showed that the Fmo3 mRNA up-regulation by 3-methylcholanthrene (3MC) and benzo(a)pyrene (BaP) but not TCDD in Hepa-1 cells is

mediated by p53 and its binding to a p53-response element in the promoter region of *Fmo3*(Celius et al., 2010).

Differentially expressed genes in the APAP autoprotection model were further analyzed usingCausal Reasoning Engine (CRE), a recently developed computational platform (O'Connor et al., 2014). CRE analysis provides hypotheses on the upstream molecular events that best explain gene expression profiles based on prior biological knowledge. CRE analysis of differentially expressed genes in APAP autoprotection study supportsan induction of the Nrf2 pathway (O'Connor et al., 2014). Additionally, the 5'-flanking region of the mouse *Fmo3* contains multiple copies of the ARE (Celius et al., 2008). Therefore, the purpose of the present study was to investigate liver *Fmo3*gene expression under oxidative stress conditions involving activation of the Nrf2-Keap1 regulatory pathway. Mice were dosed with hepatotoxicants APAP(400 mg/kg, 24 to 48h), ANIT (50 mg/kg, 2 to 48h), CCl_4(10 or 30 μL/kg, 24 and 48h) or AlOH (30 or 60 mg/kg, 6 and 24h)orunderwent sham surgery or bile duct ligation (10d). Doses selected for hepatotoxicants are based upon previous studies conducted in our laboratory resulting in oxidative stress and tissue injury. The inclusion of multiple time-points followinghepatoxicantsexposure enabled comprehensive characterization of temporal changes in *Fmo3* in relation to injury and recovery. Further, in order to investigate whether Nrf2 mediates*Fmo3* gene expression, Nrf2 KO mice were employed. APAP was used as a model toxicant in the Nrf2 KO mice study. From these experiments,it is concluded that not all hepatotoxicantsthat produce oxidative stress in mice induce liver *Fmo3* gene expression. Toxic ANIT treatment, along with the previously demonstrated APAP treatment, markedly increases *Fmo3* gene expression. While BDL increases Fmo3 mRNA expression, protein levels do not change.APAP treatment induces *Fmo3* gene expression in Nrf2 KO mice liver suggesting that the transcriptional regulation of *Fmo3* might not involve Nrf2.

2. Materials and Methods

2.1. Chemicals

Acetaminophen, alpha-naphthylisothiocyanate, carbon tetrachloride, allyl alcohol, propylene glycol and corn oil were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were of reagent grade or better and commercially available.

2.2. Animals

Male C57BL/6J mice (9- to10-week old)were purchased from Jackson Laboratories (Bar Harbor, ME) for this study. Upon arrival, mice were acclimated for one week prior to experimentation. Mice were housed in a temperature-, light- and humidity-controlled environment. Mice were fed laboratory rodent diet (Harlan Teklad 2018, Madison, WI) *ad libitum*.

2.2.1 Experimental Design 1—The primary objective of experimental design 1 was to evaluate liver Fmo3 gene expression under oxidative stress conditions involving activation of the Nrf2-Keap1 regulatory pathway. All hepatotoxicant treatments were performed after an overnight fast, and mice were re-fed 8 h after treatment. Each treatment group consisted

of six mice. For APAP treatment, mice were treated with APAP (400 mg/kg, ip) in 50% propylene glycol or vehicle only (dosing volume: 5 mL/kg). Animals were sacrificed by decapitation at 24, 48 and 72 h after treatment and blood and livers were collected for analysis. For ANIT treatment, mice were administered ANIT (50 mg/kg, po) in corn oil or vehicle only (dosing volume: 10 mL/kg). At 2, 4, 8, 12, 24 and 48 h after treatment, mice were sacrificed by decapitation for collecting samples. For CCl4 treatment, groups of mice were injected CCl4 (10 or 30 μL/kg, ip) in corn oil or vehicle only (dosing volume: 6 mL/kg) and 24 and 48 h after treatment, mice were sacrificed by decapitation to collect blood and livers for analysis. For AlOH treatment, mice were administered AlOH (30 or 60 mg/kg, ip) in saline or vehicle only (dosing volume: 10 mL/kg). Animals were sacrificed by decapitation at 6 and 24 h after treatment for sample collection and analysis. BDL liver samples were obtained from a previously described cohort (Donepudi et al., 2012). Briefly, sham or BDL surgery was performed under phenobarbital-induced anesthesia (65 mg/kg, ip). The surgeries were performed at the University of Rhode Island, College of Pharmacy animal facility with IACUC approval. Serum and liver were collected 10 d after surgery for analysis.

2.2.2 Experimental Design 2—The goal of experimental design 2 was to investigate whether Nrf2 mediateschanges in liver*Fmo3* gene expression. APAP was used as a model toxicant for Nrf2 activation.Nrf2 KO mice with a C57BL/6J background werekindly provided by Dr. Angela Slitt from the University of Rhode Island.Following overnight fasting, male Nrf2KO mice (n=6) and their wild-type counterparts (C57BL/6J) (n=6) were treated with APAP (400 mg/kg, ip) in 50% PG or vehicle (dosing volume: 5 mL/kg). Plasma and livers were collected 72h after APAP treatment for analysis.

All animal studies were performed in accordance with National Institute of Health standards and the Guide for the Care and Use of Laboratory Animals. This work was approved by the University of Connecticut's Institutional Animal Care and Use Committee.

2.3 Alanine Aminotransferase (ALT) Assay

Plasma or serum ALT activity was determined as a biochemical indicator of hepatocellular injury. Infinity ALT Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA) was used to determine ALT activity. Briefly, 100 μL of reagent was added to 10 μL serum or plasma samples, and absorbance was measured spectrophotometrically at 340 nmusing a Bio-Tek Power Wave X Spectrophotometer. ALT activity (IU/L) was determined using the molar extinction coefficient of NADH (6.3 $mM^{-1}cm^{-1}$).

2.4 Total Bile acid Assay

Total bile acids were extracted from whole liver homogenates using a t-butanol extraction method. Briefly, livers were homogenized in extraction solution (1:1, water: tbutanol) and bile acids extracted overnight at room temperature in the dark. Blood levels of total bile acid and hepatic bile acid levels were measured using a spectrophotometric bile acid assay kit (Bioquant, San Diego, CA) according to manufacturer's protocol.

2.5 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from mouse liver samples using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNAwas then reverse-transcribed into cDNA using an M-MLV RT kit (Invitrogen, Carlsbad, CA). Fmo3 mRNA expression was quantified by the ⊿⊿CT method and normalized to two housekeeping genes, β-actin and ribosomal protein S18. Data presented were normalized to β-actin. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA) and are as follow: Fmo3 forward: 5′-GGA AGA GTT GGT GAA GAC CG-3′, reverse: 5′-CCC ACA TGC TTT GAG AGG AG-3′. Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. Amplification was carried out in a 20μL reaction volume containing 8 μL diluted cDNA, Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1μM of each primer.

2.6 Preparation of Microsomal Fraction and Western Blot Analysis

Microsomes were isolated from livers as described previously (Cashman &Hanzlik, 1981; Rudraiah et al., 2014) and stored at −80°C until use. Protein concentration was determined by the method of Lowry using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). For western blot analysis, microsomal proteins (10 μg) were electrophoretically resolved using 10% polyacrylamide gels and transferred onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Membranes were blocked with 5 % non-fat powdered milk in tris buffered saline containing 0.05% tween-20(TBS-T) for 8 h. A rabbit anti-mouse Fmo3 primary antibody (GenScript USA Inc., NJ) (1:5000) was used to detect Fmo3 with β-actin as a loading control. Blots were then incubated with HRP conjugated secondary antibodies against rabbit IgG (1:2000) (Sigma-Aldrich, St Louis, MO). Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL) with visualization using GeneMate blue autoradiography film (Bioexpress, Kaysville, UT). Precision plus protein dual color standards molecular weight marker (Bio-radLaboratories, Hercules, CA) was used to identify Fmo3 protein on the blot and microsomal protein isolated from naïve female mouse liver was used as a positive control.

2.7 Enzyme Assay

Methimazole (MMI) metabolism was determined spectrophotometrically by measuring the rate of MMI *S*-oxygenation via the reaction of the oxidized product with nitro-5-thiobenzoate (TNB) to generate 5,5'-dithiobis(2-nitrobenzoate) (DTNB). The incubation mixture consisted of 50 mM sodium phosphate buffer (pH 9.0), 0.5 mM $NADP^+$, 0.5 mM glucose-6-phosphate, 1.5 IU/mL glucose-6-phosphate dehydrogenase, 0.06 mM DTNB, 0.04 mMdithiothreitol and 100 to 150 μg/mL liver microsomes isolated from mice. Reactions were initiated by the addition of different amounts of MMI (substrate), with a concentration range from 1.25 to 800 μM. Incubations were done in duplicates. The disappearance of the yellow color was measured spectrophotometrically at 412 nm and specific activity (μM/min/mg) was determined using the molar extinction coefficient of NADPH (28.2 $mM^{-1}cm^{-1}$).

2.8 Statistical Analysis

The statistical significance between groups was determined using the Student's t-test, one-way ANOVA with Dunnett's post-hoc test or two-way ANOVA followed by the Bonferroni'spost-hoc test. While Student's t-test was used to compare means of two different treatment groups, ANOVA was used to compare the means of more than two treatment groups that are normally distributed with a common variance. All statistical analysis was performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± standard error (SE), with $p<0.05$ considered statistically significant.

3. Results

3.1 Plasma ALT Activity in the Mouse Liver Injury Models

The time-points selected for the single dose APAP (400 mg/kg)treatment studyin mice were based on the previous studies that demonstrated Fmo3 mRNA and protein peaks at 48 h and 72 h, respectively(O'Connor et al., 2014; Rudraiah et al., 2014). The hepatotoxicity of a single dose APAP (400 mg/kg) at 24, 48 and 72 h as assessed by plasma ALT activity has been reported previously (Rudraiah et al., 2014). Briefly, APAP increased plasma ALT activity to 191±18 and 219±47IU/L at 24 and 48 h, respectively (mean plasma ALT activity in control mice was 25±5IU/L). Plasma ALT activity was not statistically different from vehicle controlsby 72 h, indicating recovery from APAP-induced liver injury.Plasma ALT activity in all mouse models of liver injury is shown in Figure 1. ANIT increased ALT activity at 12 h (182±9 IU/L), which continued to increase at 24 and 48 h (715±126 IU/L and 781±45 IU/L, respectively) compared to vehicle control group (9±1 IU/L). BDL for 10 d results in an elevation of ALT activity to 185±10 IU/L. A high CCl_4 dose (30 μL/kg) increased plasma ALT at 24 and 48 h to 6367±1135 and 397±111 IU/L, respectively. A low CCl_4 dose (10 μL/kg) also increased plasma ALT activity at 24 and 48 h to 757±106 and 416±68 IU/L, respectively. The greatest increase in plasma ALT with both doses of CCl_4 is observed at 24 h. While AlOHtreatment (low dose, 30 mg/kg) did not result in significant increases in ALT levels at 6 h and 24 h (36±4 and 73±24 IU/L, respectively),a high AlOHdose (60 mg/kg) results in significantly higher ALT levels at both 6 h and 24 h (153±50 and 4440±2428 IU/L, respectively). The associated histopathological damage with all hepatotoxicants has been reported previously(Aleksunes et al., 2005; Aleksunes et al., 2006; Campion et al., 2009; Donepudi et al., 2012; Rudraiah et al., 2014). With acute toxicity models, plasma ALT activity lesser than 1000 IU/L is usually associated with a minimal to mild hepatocellular damage (histological grade of 2 or less than 2), and an ALT activity higher than 1000 IU/L is associated with moderate, marked or severe hepatocellular damage (histological grade of 3, 4, or 5) (Aleksunes et al., 2008; Manautou et al., 1994). Alternatively, a 10 d BDL with an associated ALT activityof less than 200 IU/L, exhibit a chronic hepatocellular damage involving fibrosis (Donepudi et al., 2012).

3.2 Hepatic Bile Acid Concentrations in the Mouse Liver Injury Models

To determine the extent of cholestasis induced by different hepatotoxicants used in the current study, total hepatic bile acid levels were measured (Figure 2). As expected ANIT increased hepatic bile acid levels to 1.7±0.2 and 1.6±0.4 μmol/g at 24 and 48 h, respectively,

compared to 0 h vehicle control group (0.2 ± 0.02 μmol/g). BDL also increased hepatic bile acid concentrations to 19.49 ± 0.85 μmol/100 mg compared to sham operated group (10.61 ± 1.65 μmol/g). No significant changes were observed in mice treated with APAP, CCl_4 and AlOH.

3.3 Total bile Acid Concentrations in the Blood in the Mouse Liver Injury Models

Bile acid concentration in plasma is also a biomarker of cholestasis. In order to determine the extent of cholestasis, plasma bile acid levels were also quantified in all mouse models of liver injury (Figure 3). Plasma bile acid concentration increased in ANIT treated mice at 24 and 48 h to 210 ± 20 and 330 ± 128 μmol/L, respectively, compared with vehicle treated mice (2 ± 0.2 μmol/L). Bile duct ligation significantly increased serum bile acid levels to 1359 ± 142 μmol/L compared to sham operated mice (86 ± 11 μmol/L). Significant increases in plasma bile acid concentrations are seen in high dose CCl_4 treated mice at both 24 and 48 h (112 ± 15 μmol/L and 29 ± 2 μmol/L, respectively). Exposure to APAP and AlOH did not result in any significant change in plasma bile acid levels.

3.4 Hepatic Fmo3 mRNA Expression in the Mouse Liver Injury Models

Fmo3 mRNA levels were quantified by qRT-PCR and the results are presented in Figure 4. Fmo3 mRNA expression following a single dose APAP 400 mg/kg treatment has been previously reported (Rudraiah et al., 2014). Briefly, Fmo3 mRNA levels increased by 5 ± 2.6- and 23 ± 5.6-fold, at 24 and 48 h after APAP, respectively, compared to the 0 h control group. ANIT increased Fmo3 mRNA levels as early as 2 h after treatment. This increase peaked and is statistically significant only at 12 h (43 ± 10-fold increase), compared to 0 h vehicle control group. BDL also increased Fmo3 mRNA expression by 1899 ± 625-fold, compared to sham operated mice. Both the low and high dose of CCl_4 decreased Fmo3 mRNA levels at both time-points examined, and this decrease is statistically significant only at 48 h (10 μL/kg: 0.2 ± 0.06-fold and 30 μL/kg: 0.2 ± 0.08-fold). No change in liver Fmo3 mRNA levels was observed with AlOH treatment.

3.5 Fmo3 Protein Expression in the Mouse Liver Injury Models

Temporal expression of the Fmo3 protein following exposure to hepatotoxicants and BDL were quantified by western blotting. Representative blots and associated densitometric analyses are shown in Figure 5. Fmo3 protein increase following APAP treatment has been previously reported (Rudraiah et al., 2014). Although the expression of Fmo3 protein tended to increase by 1.1 ± 0.3- to 1.6 ± 0.2-fold between 24 and 72 h after APAP, the increase is statistically significant only at 72h. Consistent with Fmo3 mRNA changes evidenced with ANIT treatment, Fmo3 protein levels also tend to increase as early as 2 h. However, this increase is significant only at 12 h (3.5 ± 0.9-fold) compared to 0 h vehicle treated group. Fmo3 protein levels decreased to 0.7 ± 0.08- and 0.3 ± 0.1-fold at 24 and 48h, respectively, after exposure to ANIT. Conversely, in spite of the dramatic increase in Fmo3 mRNA levels after BDL, Fmo3 protein levels did not change. No significant changes in Fmo3 protein expression are observed with CCl_4 or AlOH treatment.

3.6 Plasma ALT Activity and Fmo3 mRNA Levels after APAP Treatment in Wild-Type and Nrf2 Knockout Mice

All models of hepatic injury used in the current study activate the Nrf2-Keap1 regulatory pathway. Furthermore, CRE analysis of differentially expressed genes in our APAP autoprotection study supportedan induction of the Nrf2 pathway (O'Connor et al., 2014). To investigate whether Nrf2 mediates*Fmo3* gene expression, APAP was used as a model toxicant for Nrf2 activation in Nrf2 KO and WT mice. Mice were administered a single 400 mg/kg APAP dose for 72 h. Dose and time-point selected were based on our single dose APAP study performed previously (Rudraiah et al., 2014), wherea 400 mg/kg APAP treatment increased Fmo3 protein levels at 72 h. Administration of 400 mg/kg APAP to male C57Bl/6J WT mice did not resultin significantlydifferent plasma ALT values at 72 h from vehicle controls. This is consistent with the previously reported results, indicating recovery from APAP-induced liver injury and increased Fmo3 protein expression in WT mice (Rudraiah et al., 2014). In contrast, plasma ALT activity is elevated at 72 h in Nrf2 KO mice receiving the same dose of APAP (176±12 IU/L) compared to vehicle control group (23±5 IU/L) (Figure 6A). This is again consistent with the literature and confirms that Nrf2 plays animportant role not only in the magnitude of toxicity, but also the degree and rate of recovery from APAP-induced liver injury. Fmo3 mRNA levels were quantified by qRT-PCR. The results in Figure 6B show that there is no change in Fmo3 mRNA levels at 72 h in wild-type mice administered 400 mg/kg APAP. This is again consistent with previous reports, where maximal Fmo3 mRNA expression is seen at 48 h after APAP administration and returns to normal by 72 h (Rudraiah et al., 2014). Notably, Fmo3 mRNA expression is significantly higherin Nrf2 KO mice 72 h after APAP treatment by 140±43-fold change compared to vehicle control group.

3.7 Fmo3 Protein Expression after APAP Treatment in Wild-Type and Nrf2 Knockout Mice

To examine the temporal changes in *Fmo3* gene expression following APAP treatment in WT and Nrf2 KO mice, Fmo3 protein levels were quantified by western blotting and by measuring catalytic activity using MMI as substrate. Representative blots and associated densitometric analysis are shown in Figure 7A. Consistent with increased Fmo3 mRNA expression in Nrf2 KO mice administered APAP (400 mg/kg) at 72 h, Fmo3 protein levels are also significantly higher (5.1±1.3-fold) compared to vehicle control group. Measuring FMO catalytic activity using MMI can also quantitate Fmo3 protein induction(Zhang et al., 2007). FMO specific activity increased significantly in Nrf2 KO livers (48±6±M/min/mg) at 72 h after APAP compared to vehicle controls of either genotype (Figure 7B).

4. Discussion

FMO3 is a microsomal enzyme involved in the oxygenation of lipophilic substrates to more polar metabolites. Substrates include nitrogen-, sulfur- and phosphorous-containing drugs and xenobiotics, and most metabolic products of Fmo3 are considered to be non-toxic(Krueger & Williams, 2005). Although FMOs were discovered in the 1960s (Miller et al., 1960) and further purified in 1972 (Ziegler & Mitchell, 1972), it was not until 2008 that Fmo3 induction by xenobiotics was demonstrated (Celius et al., 2008; Celius et al., 2010). In these studies, in spite ofa very large increase in Fmo3 mRNA level by AhR agonist

treatment, only a modest increase in protein level and function was reported. A gene array analysis performed in our laboratory also demonstrated *Fmo3* gene induction in the mouse model of APAP autoprotection(O'Connor et al., 2014). In this APAP autoprotection mouse model, we showed a significant increase in Fmo3 protein expression and function (Rudraiah et al., 2014). Constitutive Fmo3 expression in a female mouse liver is localized in the areas surrounding the periportal region (Janmohamed et al., 2004; Rudraiah et al., 2014). Following APAP exposure, the Fmo3 protein expression in APAP autoprotected livers was observed in the centrilobular regions where APAP-induced damage and/or hepatocellular compensatory proliferation is detected. Furthermore, we show that the enhanced expression of Fmo3 confers resistance against APAP-induced hepatotoxicity in mice (Rudraiah et al., 2014).

In the present study, the effect of various other hepatotoxicants on *Fmo3* gene expression in male C57BL/6J mouse liver was examined. A unifying theme for all hepatotoxicants used in our study is the oxidative stress. These models of oxidative stress used are very well studied with respect to activation of Nrf2-Keap1 regulatory pathway. This has been repeatedlydemonstrated in our laboratory and in the literature (Aleksunes et al., 2005; Aleksunes et al., 2006; Aleksunes et al., 2006; Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004; Liu et al., 2013; Randle et al., 2008; Tanaka et al., 2009). Thus, we were confident that the treatments selected result in oxidative stress and therefore the need for measuring markers of oxidative stress for each experimentalgroup of hepatotoxicity was not deemed necessary.

Coincidentally, all of the hepatotoxicants selected produce cholestasis, with the exception of AlOH(Donepudi et al., 2012; Tanaka et al., 2009; Weerachayaphorn et al., 2012; Yamazaki et al., 2013). Furthermore, perturbation of bile acid homeostasis has been demonstrated to be an early event in the pathogenesis of drug induced liver injury(Yamazaki et al., 2013). To determine whether accumulation of bile acids is a signaling event regulating *Fmo3*, we measured total hepatic and plasma bile acid levels. Consistent with the literature, ANIT and BDL increased plasma and hepatic bile acid concentrations. A high CCl_4 dose did not significantly alter liver bile acid levels, but significantly increased plasma bile acid concentration. APAP tended to increase both hepatic and plasma bile acid concentrations, but this is not statistically significant.

In general, ANIT and BDL-mediated damage significantly increased liver Fmo3 mRNA expression. While CCl_4-mediated liver injury significantly decreased Fmo3 mRNA expression, no change in expression is evidenced with AlOH. Fmo3 mRNA expressionin response to ANIT treatment precedes serum and hepatic bile acid accumulation and the maximal *Fmo3* expression (both mRNA and protein) parallels the mild hepatocellular damage observed. This observation argues against the concept that hepatic bile acid accumulation is a pre-requisite for *Fmo3* gene expression changes in the ANIT model. With BDL, unlike ANIT, Fmo3 mRNA induction is associated with higher serum and hepatic bile acid levels, but protein level does not change. Since tissues were analyzed 10 days after BDL, we do not have a sense of what the temporal relationship is between hepatic bile acid accumulation and elevated Fmo3 mRNA induction. This relationship deserves further attention based on the discussion of FXR signaling that can be found below.

Overt ANIT-induced hepatocellular damage at 24 and 48 h decreased *Fmo3* gene expression and the decease is statistically significant at 48 h compared to the 12 h ANIT-treated group. This observation with respect to the relationship between plasma ALT level and *Fmo3* gene expression is consistent with our results with APAP hepatotoxicity. We have demonstrated in our APAP autoprotection mouse model that the APAP (400 mg/kg) pretreated group as well as the autoprotected group (APAP pretreatment: 400 mg/kg, APAP challenge: 600 mg/kg) exhibit average plasma ALT values of about 250 IU/L and Fmo3 protein induction. The group that receives a toxic APAP dose of 600 mg/kg exhibits much greater average plasma ALT activity of about 1600 IU/L, but no *Fmo3* gene induction (Rudraiah et al., 2014).

One key feature that is consistent with APAP-, BDL- and ANIT-induced liver injury and enhanced *Fmo3* gene expression is the magnitude in plasma ALT elevations. Mild ALT elevation is the common feature for all three models where Fmo3 mRNA induction is observed. Although BDL is a chronic injury model, it well known that the ALT values do not correlate well with the severity of liver damage particularly during fibrotic hepatocellular necrosis (Kallai et al., 1964). A similar clinical feature is also seen in cases of primary biliary cirrhosis (BDL models primary biliary cirrhosis in humans) (Hohenester et al., 2009). The lack of Fmo3 protein detection during BDL may be due to signal dilution by fibrotic liver tissue. It is also possible that under oxidative stress conditions some proteins involved in translation are oxidized *in vivo* inhibiting translation (Shenton et al., 2006). To add to this complexity, in spite of lower plasma ALT activity at 24 h (low dose CCl_4), and 48 h (both high and low dose CCl_4) with CCl_4, there is down-regulation of *Fmo3* gene expression. In AlOH-treated livers, lower plasma ALT activity did not show any change in *Fmo3* gene expression. This is suggestive of an unknown underlying mechanism unique to APAP, ANIT and BDL, which is contributing to Fmo3 mRNA induction. Studies are necessary to investigate the regional distribution of Fmo3 protein expression during ANIT- and BDL-induced liver injury to determine whether the protein expression in these models is localized to the portal vein, where ANIT- and BDL-induced hepatotoxicity is confined. Collectively, these data suggest that there is a threshold for degree of hepatic injury that results in increased *Fmo3* gene and protein expression.More specifically,these data suggest that acute moderate or mild hepatotoxicity is optional for *Fmo3* induction, while chronic, severe hepatotoxicity is not.

As discussed before, all models of hepatic injury used in the current study result in hepatic oxidative stress and activate the Nrf2-Keap1 regulatory pathway. Importantly, CRE analysis of differentially expressed genes in our APAP autoprotection study supportedan induction of the Nrf2 pathway (O'Connor et al., 2014). Additionally, Celius *et al.* (2008) showed that the 5'-flanking region of the mouse *Fmo3* contains multiple copies of the ARE (Celius et al., 2008). Promoter analysis of mouse *Fmo3* promoter (7 kb length)using MatInspector software (Genomatix, Munich, Germany), also showed an ARE at about 3 kb from the transcription start site (data not shown). We also found two other binding sites for Bach1, at 600 bp and 2 kb from the *Fmo3* transcription start site (data not shown). Bach1 is a regulatory mediator of Nrf2, in that it is a transcription repressor. Bach1 heterodimerizes with small Maf proteins in the absence of cellular stress and represses gene expression. In

the presence of oxidative stress, Bach1 is released from the Maf proteins and is replaced by Nrf2(Kaspar et al., 2009). Thus, using APAP as a model toxicant for Nrf2 activation, *Fmo3* gene expression was evaluated inNrf2 KO mice. Compared to APAP-treated wild-type mice, Nrf2 KO mice exhibit persistent and significant hepatocellular damage 72 h after APAP administration. Nrf2 KO mice are not only more susceptible to APAP-induced hepatocellular necrosis, but also fail to recover from injury as rapidly as the WT mice. This is consistent with the literature that describes Nrf2 as a master regulator of many cytoprotective genes involved in APAP-induced hepatotoxicity (Aleksunes et al., 2005; Aleksunes et al., 2006; Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004). Finally, *Fmo3* gene induction by APAP treatment in Nrf2 null mice suggests that the transcriptional regulation of *Fmo3*does not involve this transcription factor. The persistent mild hepatocellular injury and oxidative stress in Nrf2 KO mice most likely activates other signaling mechanisms involved in *Fmo3* gene induction.

Future studies will investigate the role of other nuclear receptors in *Fmo3* gene induction. Particularly, farnesoid X receptor (FXR) role in *Fmo3* gene induction during APAP hepatotoxicity is worth investigating. FXR is one of the major bile acid sensors in the liver (Chiang, 2002) and plays a protective role during cholestasis development. Recently, it is shown that activation of FXR induces Fmo3 protein function (Bennett et al., 2013). Activation of FXR also provides protection against APAP-induced hepatotoxicity(F. Y. Lee et al., 2010). In addition, we discovered three binding sites for farnesoid X receptor-response element (FXRE) at about 1.6, 2.1 and 3.3 kb from the transcription start site on the mouse *Fmo3* promoter (data not shown). Although, Pregnane X receptor (PXR) is also a bile acid sensor, no binding sites for PXR were found on promoter analysis and furthermore, PXR activation sensitizes APAP-induced hepatotoxicity (Cheng et al., 2009; Guo et al., 2004).

In conclusion, this study comprehensively characterizes for the first time temporal changes in *Fmo3* gene expression during different conditions known to impart hepatic oxidative stress. In particular, we show that toxic ANIT and BDL significantly alter Fmo3 mRNA expression, but only Fmo3 protein with ANIT. The reason for this discrepancy is not yet clear or easy to rationalize. It is possible that *Fmo3* is also protective during other chemical-induced liver injury including ANIT. Even though the exact mechanism of *Fmo3* gene expression or its role in protecting against toxicant-induced liver injury is not yet clear, the observed toxicity threshold for *Fmo3* gene expression is intriguing. This work advances the lack of knowledge with regard to the inducibility of *Fmo3* and its potential protective role in drug-induced liver injury.

Acknowledgements

Abbreviations

Fmo3	Flavin-containing monoxygenase-3
APAP	Acetaminophen

ANIT	Alpha-naphthylisothiocyanate
BDL	Bile duct ligation
CCl$_4$	Carbon tetrachloride
AlOH	Allyl alcohol
Nrf2	Nuclear factor (erythroid-derived 2)-like 2

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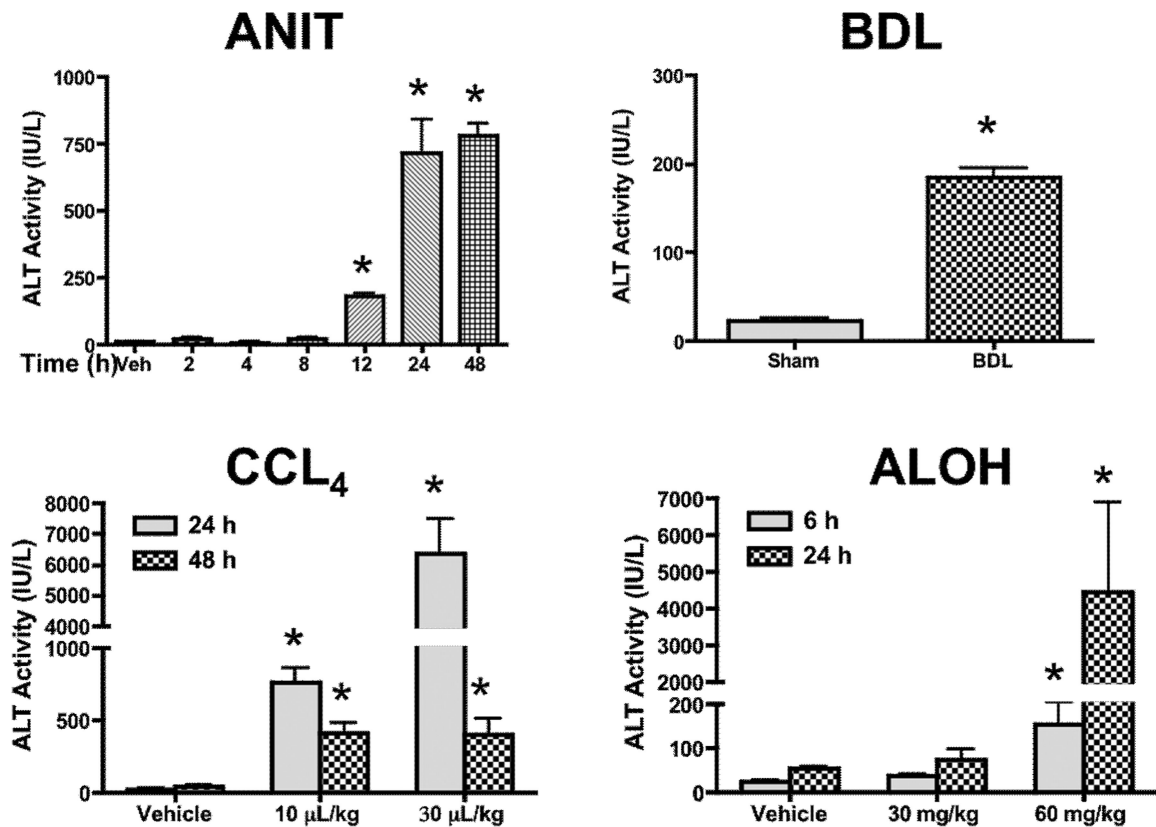
Figure 1. Plasma ALT activity in mice treated with hepatotoxicants or BDL
Male C57BL/6J mice (n=6 per group) were treated with ANIT (50 mg/kg, po, 2,4,8,12,24 & 48 h), CCl$_4$ (10 & 30 μL/kg, ip, 24 & 48h), AlOH (30 & 60 mg/kg,ip, 6 & 24 h) or BDL (10 d). Plasma was collected from mice at various time-points following hepatotoxicant treatment or the appropriate vehicle. The data are presented as mean plasma ALT (IU/L) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference (p < 0.05) between vehicle-treated and hepatotoxicant-treated group.
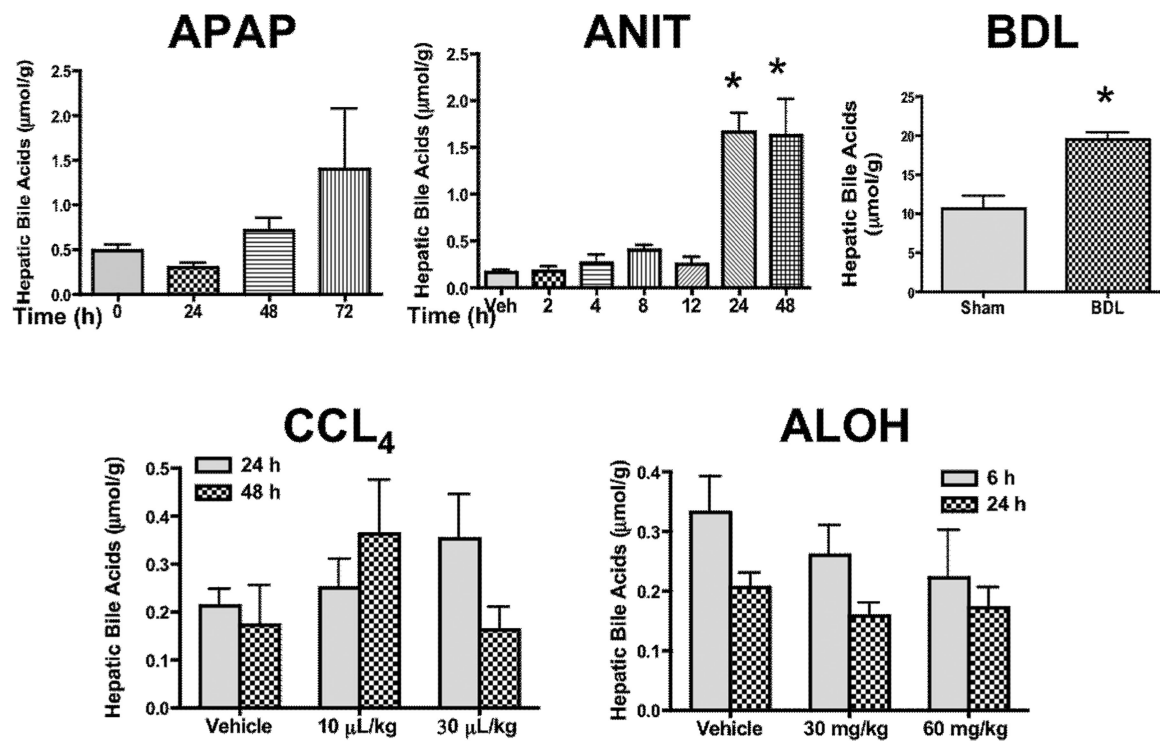
Figure 2. Hepatic bile acid levels after hepatotoxicant administration or BDL
Livers were collected at the end of each study (24, 48 & 72h for APAP; 2,4,8,12,24 & 48h for ANIT; 10d for BDL; 24 & 48h for CCl$_4$; and 6 & 24h for AlOH). Liver total bile acids were measured spectrophotometrically using a commercial bile acid assay kit. Hepatic total bile acids are expressed as mean hepatic bile acids (μmol/g) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for one-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference (p < 0.05) between vehicle-treated and hepatotoxicant-treated or BDL group.
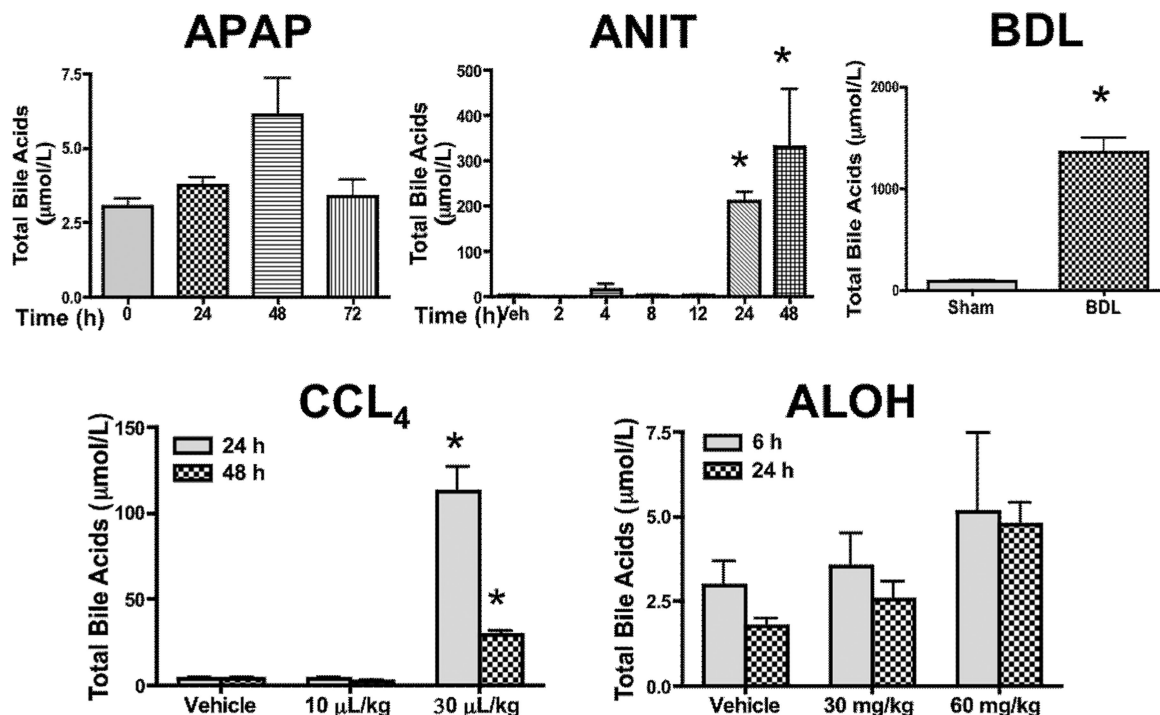
Figure 3. Blood levels of TotalBile acid in the mouse models of liver injury

Mice (n=6 per group) were administered APAP (400 mg/kg, ip, 24, 48 & 72h), ANIT (50 mg/kg, po, 2,4,8,12,24 & 48h), CCl$_4$(10 & 30 μL/kg, ip, 24 & 48 h), AlOH (30 & 60 mg/kg, ip, 6 & 24h) or BDL (10 d).Blood was collected and the plasma or serum (for BDL) was isolated by centrifugation. Total bile acid levels were measured spectrophotometrically using a commercial bile acid assay kit. Total bile acids are expressed as mean total bile acids (μmol/L) ± SE. Student's t-test,one-way ANOVA,or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for one-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.
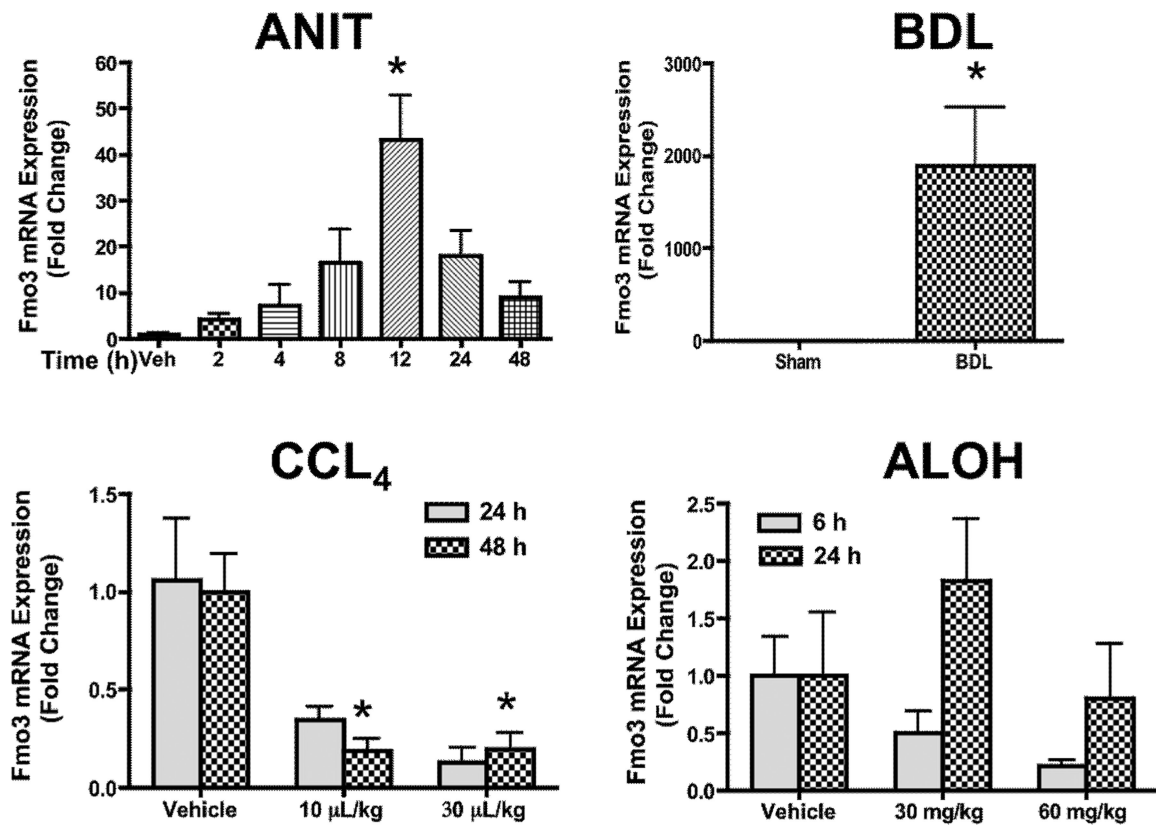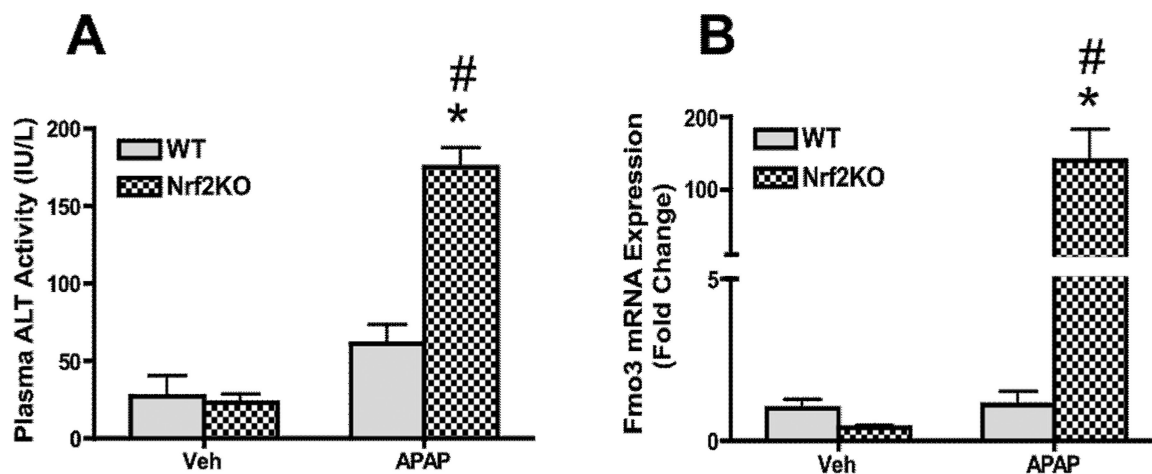
Figure 4. Quantitative RT-PCR analysis of liver Fmo3 transcripts after hepatotoxicant treatment or BDL

Livers were collected from mice (n=6) sacrificed at respective time-points (2,4,8,12,24 & 48h for ANIT; 24 & 48h for CCl_4; 6 & 24h for AlOH; and 10 d for BDL). RNA was isolated and cDNA was made using a commercial MMLV-RT kit. The cDNA samples were analyzed by quantitative RT-PCR using Fmo3 mouse-specific primers. Gene expression was normalized to the housekeeping gene β-actin. Fmo3 mRNA expression is presented as mean Fold Change ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.
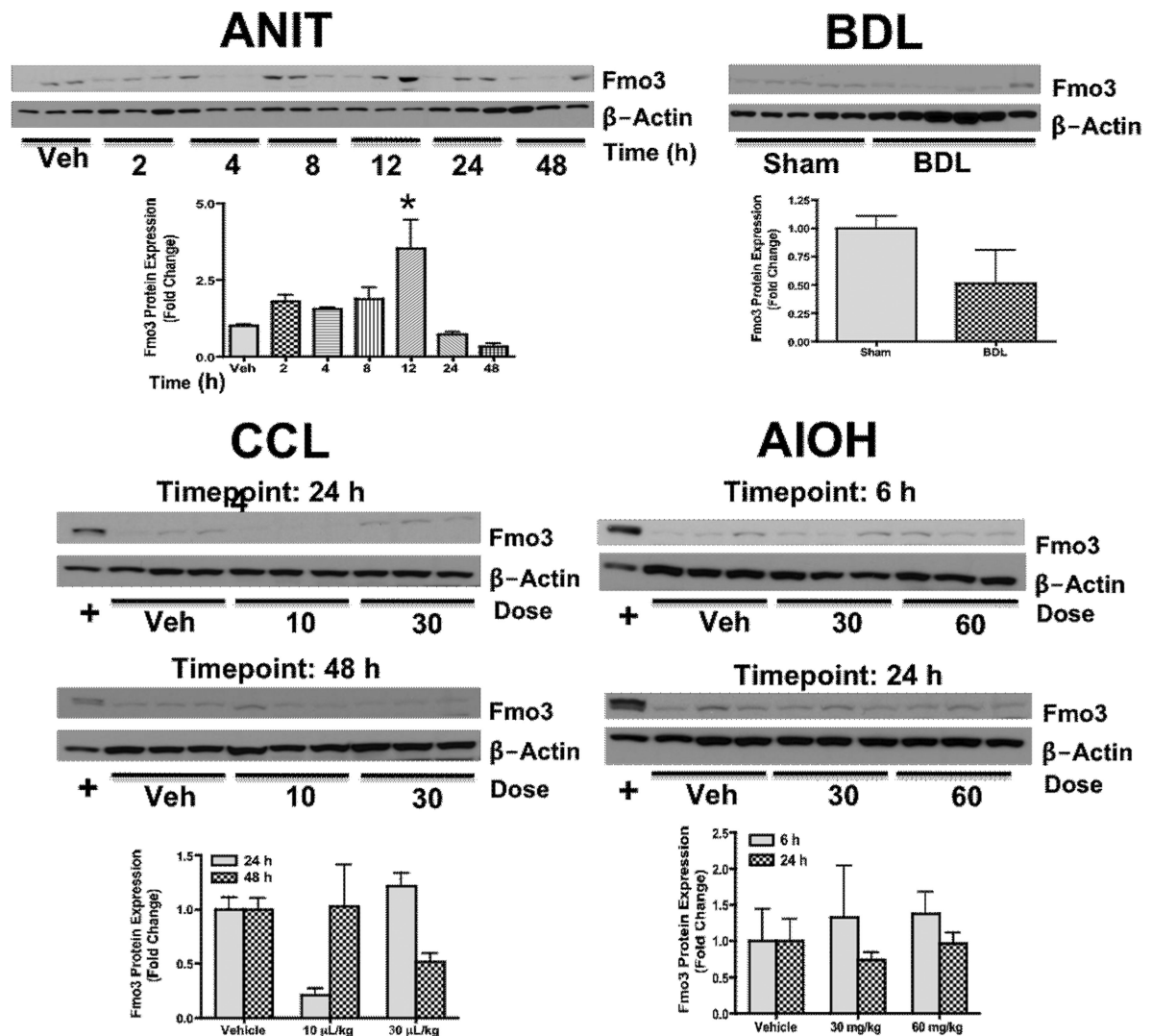
Figure 5. Analysis of liver Fmo3 protein expression in the mouse models of liver injury by western blotting

Western immunoblots for Fmo3 were performed using liver microsomes from control and hepatotoxicant-treated or BDL mice. A custom-made rabbit anti-mouse Fmo3 primary antibody, described in *Materials and Methods* was used to detect Fmo3. Fmo3 protein levels were normalized to β-actin loading control. Microsomal proteins isolated from naïve female mouse liver were used as a positive control (indicated by "+" sign). The data are presented as blots and as mean Fmo3 protein expression (Fold Change) ± SE. One-way ANOVA, t-test or two-way ANOVA was performed, appropriately, followed by the Dunnett's posttest for One-way ANOVA and the Bonferroni posttest for two-way ANOVA. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated and hepatotoxicant-treated or BDL group.

Figure 6. Plasma ALT activity and quantitative RT-PCR analysis of liver Fmo3 transcripts following a single dose APAP treatment in wild-type and Nrf2knockout mice
Plasma and livers were collected from mice 72 h following APAP (400 mg/kg) or vehicle treatment. **(A)** The data are presented as mean plasma ALT (IU/L) ± SE. **(B)** RNA was isolated from livers andcDNA samples were analyzed by quantitative RT-PCR using Fmo3 mouse-specific primers. Gene expression was normalized to the housekeeping gene β-actin. Fmo3 mRNA expression are presented as mean Fold Change ± SE. Oneway ANOVA was performed followed by the Dunnett's post-test. Asterisks (*) represent a statistical difference (p < 0.05) between vehicle-treated group and APAP-treated group and hash (#) represent a statistical difference (p<0.05) compared with APAP-treated wild-type mice.
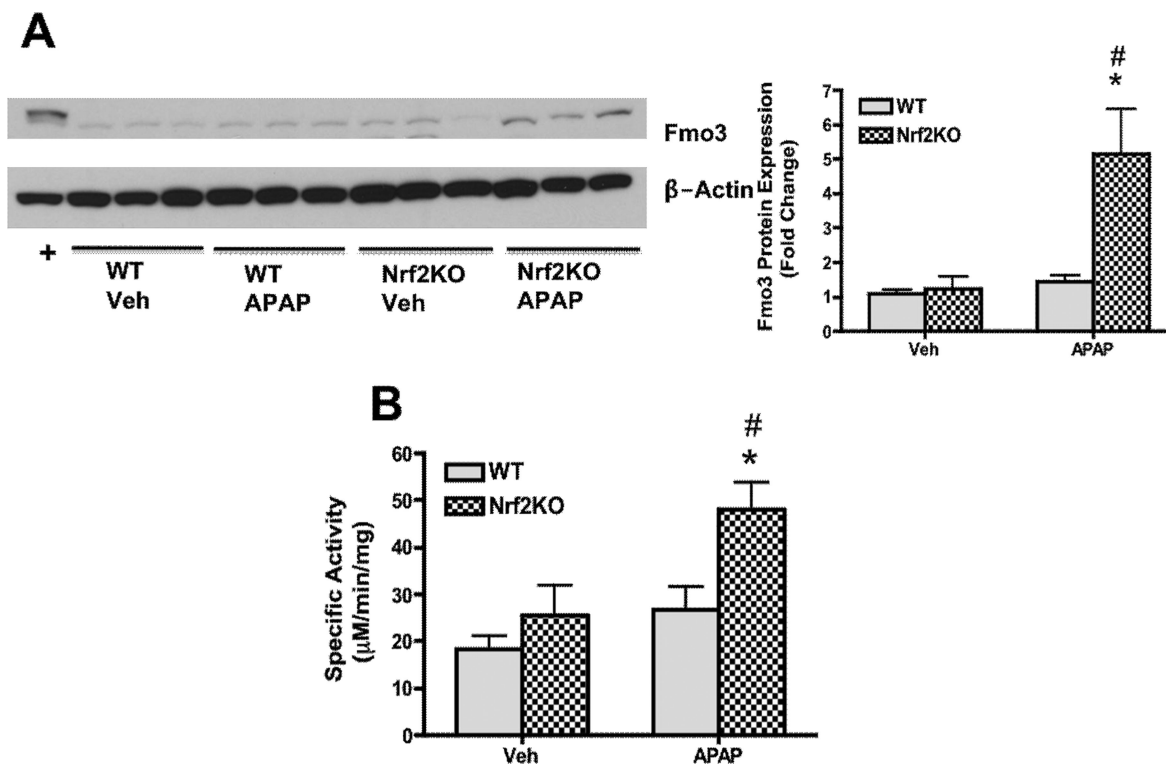
Figure 7. Analysis of liver Fmo3 protein expression following a single dose APAP treatment in wild-type and Nrf2knockout mice by western blotting and enzyme activity assay

After overnight fasting, groups of wild-type and Nrf2 knockout mice received a single dose of 400 mg/kg APAP or vehicle. Livers were collected 72 h following APAP or vehicle treatments. Western blot for Fmo3 was performed using liver microsomes from control and APAP-treated mice. Equal protein loading (10 μg protein/lane) was confirmed by detection of β-actin. Microsomal proteins isolated from naïve female mouse liver were used as a positive control indicated by "+" sign. The data are presented as blots and as mean Fmo3 protein expression (Fold Change) ± SE **(A)**. FMO activity was measured in liver microsomes from control and APAP-treated mice using methimazole as substrate. Data are presented as mean Specific Activity (μM/min/mg) ± SE **(B)**. Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle-treated group and APAP-treated group and hash (#) represent a statistical difference ($p<0.05$) compared with APAP-treated wild-type mice.